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Original article

Development of bovine embryos *in vitro* following oocyte maturation under defined conditions

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Summary — A total of 4 615 immature bovine oocytes were used in a series of experiments aimed at the systematic evaluation of the role of the different components of our *in vitro* maturation (IVM) medium in imparting developmental competence to the oocytes. The results clearly demonstrate that both tissue culture medium 199 (M199) and synthetic oviduct fluid (SOF) are capable of supporting the IVM of bovine oocytes at high rates in the absence of macromolecular supplements, as evidenced by subsequent development to the blastocyst stage (20 and 25%, respectively). However, both were significantly lower than the control (containing 10% fetal calf serum, 5 μg/ml pLH (porcine luteinizing hormone), 1 μg/ml pFSH (porcine follicle-stimulating hormone), and 1 μg/ml-17β-estradiol, E2) in terms of blastocyst yield. Inclusion of bovine serum albumin (3 mg/ml) was not beneficial and in fact significantly depressed development when added to SOF. It was shown that the advantage of the control conditions over unsupplemented M199 and SOF was entirely attributable to the presence of serum and that in the absence of serum the inclusion of the above hormone cocktail significantly depressed post-cleavage development. When used individually, neither LH (50, 500, 5 000 ng/ml) nor FSH (10, 100, 1 000 ng/ml) improved development over M199 alone. In conclusion, we now have a simple maturation system, using a fully defined medium (M199) in which the search for factors improving the cytoplasmic competence acquisition of maturing cattle oocytes will be possible.

IVM / IVF / oocyte / medium / bovine

Résumé — Développement des embryons bovins *in vitro* suivant la maturation de l'ovocyte dans des conditions bien définies. *Un total de 4 615 ovocytes a été utilisé dans une série d'expériences destinées à évaluer l'effet respectif des différents composants du milieu de maturation* in vitro *(MIV) sur l'acquisition par les ovocytes bovins de leur compétence à se développer* in vitro *après fécondation (compétence cytoplasmique). Les résultats ont montré clairement que le milieu 199 (M199) et le fluide d'oviducte synthétique (SOF) sont capables à eux seuls de supporter la MIV d'ovocytes bovins. Cependant, les taux de développement jusqu'au stade de blastocyste observés après maturation dans ces milieux purs (20 et 25% respectivement) sont significativement inférieurs à ceux qui sont obtenus après maturation dans le milieu contrôle (M199 additionné de 10% de sérum bovin fœtal, 5 μg/ml de pLH, 1 μg/ml de pFSH et 1 μg/ml de 17β-œstradiol). L'ajout d'albumine bovine (3 mg/ml) à ces milieux*

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n'améliore pas la qualité de la maturation. Au contraire, l'albumine diminue le taux de développement lorsqu'elle est ajoutée au SOF pendant la maturation. Nous avons montré que l'entièreté de l'effet bénéfique du milieu contrôle résidait dans la présence de sérum. En effet, les hormones (FSH et LH) ajoutées ensemble ou séparément (à différentes concentrations) au milieu de maturation n'améliorent pas la compétence cytoplasmique des ovocytes. En conclusion, nous disposons maintenant d'un système simple dans lequel nous allons pouvoir rechercher, parmi les constituants du sérum, ceux qui agissent sur la compétence cytoplasmique de l'ovocyte bovin.

MIV / FIV / ovocyte / milieu de culture / bovin

INTRODUCTION

In recent years substantial progress has been made in the development of procedures for the *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of bovine embryos (for reviews, see Brackett and Zuelke, 1993; Trounson *et al,* 1994). However, further improvements are necessary to maximize embryo production. In most laboratories, the production of viable embryos plateaus out at 20–30% of inseminated oocytes in spite of numerous variations on the basic technique. It would appear that not all oocytes are fully capable of responding to the maturation conditions to which they are exposed. The shortfall in developmental ability is probably due to inadequacies in cytoplasmic maturation.

Much research in the area of *in vitro* cattle embryo production has been directed towards the identification of factors involved in imparting developmental competence to the oocyte following IVM. Factors important during IVM include: 1) the hormonal environment of the oocyte prior to and during IVM (Moor and Trounson, 1977; Zhang and Armstrong, 1989); 2) the presence of serum in the IVM medium (Downs *et al,* 1986); and 3) the association of follicular or cumulus cells (*ie* somatic factors) with the maturing oocyte (Vanderhyden and Armstrong, 1989). These factors may play a role in supporting oocyte cytoplasmic maturation, which is critical for subsequent fertilization and development.

It has been well established that culture conditions for IVM of mammalian oocytes can significantly influence the subsequent development of such oocytes (mouse: Eppig *et al,* 1990; Van de Sandt *et al,* 1990; pig: Yoshida *et al,* 1992; cattle: Fassi-Fihri *et al,* 1991; Keefer *et al,* 1991; Ectors *et al,* 1992; Rose and Bavister, 1992). However, the specific mechanisms by which various IVM medium components affect subsequent development remain speculative. The situation is further confused by the chemical heterogeneity introduced into the medium by serum.

The present series of experiments involved the use of 4 615 immature bovine oocytes to systematically examine the role of the components of our IVM medium to determine their relative importance in imparting developmental competence to the oocytes.

MATERIALS AND METHODS

General procedures

Oocyte collection and maturation

Oocytes were obtained by aspiration of 2–6 mm follicles of ovaries from slaughtered cows. All oocytes completely surrounded by unexpanded cumulus cells were used (average 5.7 oocytes per ovary); they were washed 4 times in modified phosphate-buffered saline (PBS) (supplemented with pyruvate 36 mg/l, gentamycin and 0.5 mg/ml bovine serum albumin, BSA Sigma

fraction V, cat # A9647). Groups of up to 50 oocytes were transferred to 4-well plates (Nunc, Roskilde, Denmark) containing 500 µl of medium for 24 h maturation at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity.

The control medium used for maturation throughout all experiments consisted of tissue culture medium 199 (M199, Gibco, Paisley, Scotland, cat #041–01150H) supplemented with 10% heat-treated fetal calf serum (FCS, Gibco cat #011–06180H), 5 µg porcine luteinizing hormone (pLH) per ml, 1 µg porcine follicle-stimulating hormone (pFSH) per ml and 1 µg 17β-estradiol (E2) ml. pLH and pFSH were supplied by JF Beckers, Liège, Belgium).

In vitro fertilization

Following maturation, cumulus expansion was visually assessed under a stereo-microscope. Oocytes were then washed 4 times in PBS and once in fertilization medium before being transferred in groups of up to 100 into 4-well plates containing 250 µl fertilization medium (TALP, containing 10 µg heparin-sodium salt (167 U/mg, Calbiochem, San Diego, CA) per ml) per well. Motile spermatozoa were obtained by centrifugation of frozen-thawed spermatozoa on a Percoll (Pharmacia, Uppsala, Sweden) discontinuous density gradient (2 ml at 45% over 2 ml at 90%) for 20–30 min at 700 *g* at room temperature. The same ejaculate from one bull was used throughout all experiments. Viable spermatozoa, collected at the bottom of the 90% fraction were washed in TALP and pelleted by centrifugation at 100 *g* for 10 min at room temperature. Spermatozoa were counted in a hemocytometer and diluted in the appropriate volume of TALP to give a concentration of 4 x 10^6 spermatozoa/ml; 250 µl of this suspension was added to each fertilization well to obtain a final concentration of 2 x 10^6 spermatozoa/ml. Plates were then incubated for 18–21 h in 5% CO_2 in humidified air at 39°C.

In vitro culture

For all experiments culture took place in modified synthetic oviduct fluid medium (SOF; Takahashi and First, 1992) under paraffin oil in a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 39°C. Cumulus cells were removed from presumptive zygotes by vortexing for 2 min in 2 ml PBS. The zygotes were subsequently washed twice in PBS and twice in SOF before being transferred to the culture droplets (1 zygote/µl medium). Fetal calf serum was added (10% v/v) 24 h after placement in culture (*ie* 48 h post-fertilization). Cleavage was assessed 48 h after placement in culture (% non-cleaved, 2–4 cells, 5–8 cells). The number of embryos developing to the expanded blastocyst stage was assessed on days 6–8 of culture. Hatching was recorded on day 8 of culture. It should be noted that while this hatching rate was sufficient for comparisons among treatments it should not be considered as representative of the total hatching rate in our system as in cases where culture was continued beyond day 8 additional hatching occurred.

While every effort was made to totally remove cumulus cells prior to placement in culture, this was not achieved in all cases, with a monolayer being evident in the culture droplet at the end of the culture period. Preliminary experiments would suggest that these cells are not essential for embryo development.

Experiment 1: effect of basal medium used during IVM on subsequent development

In an effort to see if the SOF we used for culture of post-fertilization embryos could support IVM without adversely affecting subsequent development, oocytes were matured in either M199, SOF (as used for embryo culture, *ie* with 3 mg/ml BSA, but without serum) or control medium (see above) *(Experiment 1a)*.

To ensure that the effect of the SOF above was not due solely to the presence of BSA, a second experiment was performed in which BSA was added to M199 at the same concentration (3 mg/ml) during maturation *(Experiment 1b)*.

Experiment 2: effect of addition of hormones and/or serum to the IVM medium on subsequent development

(a) M199 *vs* M199 + H *vs* M199 + FCS *vs* Control

(b) SOF *vs* SOF + H *vs* SOF + FCS *vs* SOF + H + FCS *vs* Control

Oocytes were matured in either M199 or SOF. This was used supplemented with (i) H: 5 µg

LH/ml, 1.0 μg pFSH/ml, 1 μg 17β-estradiol; (ii) 10% heat-treated FCS; or (iii) a combination of (i) and (ii).

Experiment 3: effect of addition of pFSH to IVM medium on subsequent development

To assess the effect of FSH on development, oocytes were matured in M199 alone or in the presence of pFSH at 10, 100 or 1 000 ng/ml.

Experiment 4: effect of pLH addition to IVM medium on subsequent development

To assess the effect of LH on development, oocytes were matured in M199 alone or in the presence of pLH at 50, 500, or 5 000 ng/ml.

RESULTS

Experiment 1a

The results are shown in table I. In the majority of cases with M199 and in all cases with SOF, oocytes did not exhibit cumulus expansion following IVM. Oocytes matured in control medium always exhibited pronounced expansion. There was no significant difference in terms of cleavage rate, % 5–8 cells or hatching rate between any of the 3 groups. While there was no significant differences between M199 and SOF in terms of blastocyst yield, both were significantly lower than the control ($P < 0.05$) expressed as a percentage of oocytes inseminated (20 and 25 *vs* 37%, respectively) or oocytes cleaved (24 and 31 *vs* 44%, respectively).

Experiment 1b

The results are shown in table II. Addition of BSA to M199 had no effect on any of the parameters studied. In contrast, addition of BSA to SOF significantly reduced the blastocyst yield, taken either as a percentage of oocytes inseminated (31 *vs* 17%, respectively) or oocytes cleaved (40 *vs* 25%, respectively). Both M199 and SOF, either in the presence or absence of BSA were significantly lower than the control in terms of blastocyst yield.

Experiment 2

The results are shown in tables III and IV. Addition of hormones and serum, either alone or in combination with M199 and SOF had a marked positive effect on cumulus

Table I. Effect of medium used during IVM on subsequent bovine embryo development.

Treatment	*Ova* *	*No embryos cleaved (%)*	*No embryos 5–8 cell (%)*	*No embryos blastocysts on day 8 (%)*	*% blastocysts /cleaved*	*No embryos hatched on day 8 (% hatching)*
M199	168	136 (81)	84 (50)	33[a] (20)	24[a]	13 (39)
SOF + BSA	185	146 (79)	90 (49)	46[a] (25)	31[a]	20 (43)
Control	191	159 (83)	105 (55)	70[b] (37)	44[b]	31 (44)

[a,b,c] Values in the same column with different superscripts differ significantly (χ^2, $P < 0.05$). * Total of 3 replicates. Control: M199 supplemented with 5 μg/ml LH + 1 μg/ml FSH + 1 μg/ml E_2 + 10% FCS.

Table II. Effect of BSA inclusion in IVM medium on subsequent bovine embryo development.

Treatment	*BSA*	*Ova **	*No embryos cleaved (%)*	*No embryos 5–8 cell (%)*	*No embryos blastocysts on day 8 (%)*	*% blastocysts /cleaved*
M199	–	119	78^{a} (65)	51 (43)	24^{ab} (20)	31^{ab}
	+	131	96^{ab} (73)	61 (47)	31^{ab} (24)	32^{ab}
SOF	–	106	83^{b} (78)	48 (45)	33^{ac} (31)	40^{ac}
	+	124	83^{ab} (67)	50 (40)	21^{b} (17)	25^{b}
Control		118	85^{ab} (72)	51 (43)	44^{c} (37)	52^{c}

^{a,b,c} Values in the same column with different superscripts differ significantly (χ^2, $P < 0.05$). * Total of replicates. Control: M199 supplemented with 5 μg/ml LH + 1 μg/ml FSH + 1 μg/ml E_2 + 10% FCS.

Table III. Effect of addition of serum and/or hormones to M199 during IVM on subsequent bovine embryo development.

Treatment	*Ova **	*No embryos cleaved (%)*	*No embryos 5–8 cell (%)*	*No embryos blastocysts on day 8 (%)*	*% blastocysts /cleaved*
M199	159	130^{a} (82)	82 (52)	44^{a} (28)	34^{ab}
M199 + H	157	118^{ac} (75)	80 (51)	27^{b} (17)	23^{b}
M199 + S	171	118^{bc} (69)	89 (52)	53^{a} (31)	45^{a}
Control	213	163^{ac} (76)	119 (56)	60^{a} (28)	37^{a}

^{a,b,c} Values in the same column with different superscripts differ significantly (χ^2, $P < 0.05$). * Total of 4 replicates. Control: M199 supplemented with 5 μg/ml LH + 1 μg/ml FSH + 1 μg/ml E_2 + 10% FCS.

expansion. Addition of hormones to M199 significantly depressed the yield of blastocysts after IVF compared with M199 alone. This effect of hormones was removed in the presence of serum. There was no difference, however, between IVM in M199 alone, M199 + serum, or the control.

Addition of hormones to SOF during IVM significantly depressed the subsequent blastocyst hatching rate. As observed with M199, the addition of serum completely blocked this effect.

Experiment 3

The results are shown in table V. FSH at any of the concentrations studied significantly improved cumulus expansion compared with M199 alone. Addition of 100 ng/ml FSH significantly increased the cleavage rate over that of M199 alone. However, at higher (1 000 ng/ml) or lower (10 ng/ml) concentrations there was no difference. Addition of FSH at any of the concentrations studied had no effect on blastocyst

Table IV. Effect of addition of serum and/or hormones to SOF during IVM on subsequent bovine embryo development.

Treatment	*Ova* *	*No embryos cleaved (%)*	*No embryos 5–8 cell (%)*	*No embryos blastocysts on day 8 (%)*	*% blastocysts /cleaved*	*No embryos hatched on day 8 (% hatching)*
SOF	180	144 (80)	105^{a} (58)	34^{a} (19)	24^{a}	14^{ac} (41)
SOF + H	160	130 (81)	85^{ab} (53)	35^{a} (22)	27^{ac}	8^{a} (23)
SOF + FCS	166	130 (78)	82^{ab} (49)	30^{a} (18)	23^{a}	15^{bc} (50)
SOF + H + FCS	162	118 (73)	75^{b} (46)	43^{ab} (27)	36^{bcd}	22^{bc} (51)
Control	163	126 (77)	91^{ab} (56)	54^{b} (33)	43^{d}	26^{bc} (48)

^{a,b,c} Values in the same column with different superscripts differ significantly (χ^2, $P < 0.05$). * Total of 4 replicates. Control: M199 supplemented with 5 μg/ml LH + 1 μg/ml FSH + 1 μg/ml E_2 + 10% FCS.

Table V. Effect of pFSH addition to IVM medium on subsequent bovine embryo development.

Treatment	*Ova* *	*No embryos cleaved (%)*	*No embryos 5–8 cell (%)*	*No embryos blastocysts on day 8 (%)*	*% blastocysts /cleaved*	*No embryos hatched on day 8 (% hatching)*
M199	174	140^{a} (80)	87 (50)	34^{a} (19)	24^{a}	15 (44)
10 ng	174	151^{ab} (87)	100 (57)	31^{a} (18)	20^{a}	10^{a} (32)
100 ng	172	153^{b} (89)	98 (57)	43 (25)	28^{ab}	12^{a} (28)
1 000 ng	162	135^{ab} (83)	80 (49)	31^{a} (19)	23^{a}	16^{b} (52)
Control	176	146^{ab} (83)	91 (52)	55^{b} (31)	38^{b}	30^{b} (54)

^{a,b,c} Values in the same column with different superscripts differ significantly (χ^2, $P < 0.05$). * Total of 3 replicates. Control: M199 supplemented with 5 μg/ml LH + 1 μg/ml FSH + 1 μg/ml E_2 + 10% FCS.

yield compared to M199 alone. All concentrations except 100 ng FSH were significantly lower than the control in terms of blastocyst yield.

Experiment 4

The results are shown in table VI. Addition of LH at concentrations in excess of 50 ng/ml significantly improved cumulus expansion compared to M199 alone. There was no significant difference in terms of cleavage rate amongst any of the 5 treatment groups. There was not much variation in the %5–8 cell embryos at 72 hpi, except between 50 ng LH and 5 000 ng LH ($P < 0.05$, 39 *vs* 51%, respectively). In terms of blastocyst yield, expressed either as a percentage of oocytes inseminated or of oocytes cleaved, there was no difference between maturation in M199 alone or in the presence of LH at any of the concentrations studied. How-

Table VI. Effect of pLH addition during IVM on subsequent bovine embryo development.

Treatment	*N*	*Ova*	*No embryos cleaved (%)*	*No embryos 5–8 cell (%)*	*No embryos blastocysts on day 8 (%)*	*% blastocysts /cleaved*
M199	4	167	167 (72)	79^{ab} (47)	40^{a} (24)	33^{a}
50 ng	3	155	109 (70)	60^{a} (39)	33^{a} (21)	30^{a}
500 ng	5	263	194 (74)	121^{ab} (46)	56^{a} (21)	29^{a}
5 000 ng	5	280	218 (78)	143^{b} (51)	75^{a} (27)	34^{a}
Control	5	219	172 (78)	101^{ab} (46)	87^{b} (40)	51^{b}

^{a,b} Values in the same column with different superscripts differ significantly (χ^2, $P < 0.05$). N: number of replicates. Control: M199 supplemented with 5 µg/ml LH + 1 µg/ml FSH + 1 µg/ml E_2 + 10% FCS.

ever, all were significantly lower than the control for both parameters.

DISCUSSION

Two principal factors known to influence the IVM process in many species are protein and hormonal media supplements. The complex culture medium TCM 199 buffered with bicarbonate or HEPES and supplemented with various sera and/or gonadotropin (FSH, LH) and/or steroid (E_2) hormones has been the most widely used culture medium for the study of bovine oocyte maturation. It has also been reported that follicular somatic cells affect subsequent *in vitro* development when oocytes are cultured with these cells during IVM (Staigmiller and Moor, 1984; Critser *et al,* 1986; Lu and Gordon, 1987; Lutterbach *et al,* 1987; Faundez *et al,* 1988; Chikamatsu *et al,* 1989). However, as pointed out by Downs *et al* (1991), the presence of unidentified components or contaminants in these supplements makes it difficult to know with certainty whether the response of the oocyte is attributable to a certain hormonal or chemical manipulation during culture or is, at least in part, influenced by unknown components of the supplement.

The results of *Experiment 1* demonstrate that both M199 and SOF are capable of supporting the IVM of bovine oocytes in the absence of macromolecular supplements as evidenced by subsequent development, although to a lower extent than control conditions in terms of blastocyst yield. It was also clearly demonstrated that BSA is not a necessary supplement and in fact depresses blastocysts yield when added to SOF.

It has been shown that cumulus expansion is not necessary to achieve normal nuclear configurations during IVM (Sirard *et al,* 1988). The present study confirms this finding by taking the results further along the developmental axis, *ie* to the blastocyst stage. In the majority of experiments using M199 alone for IVM, and in all those using SOF alone for IVM, no cumulus expansion was observed; however, subsequent blastocyst yields were unaffected. In some instances, oocytes exhibited pronounced expansion in M199 alone. The reasons for the inconsistency are unclear but are probably partly related to the number of oocytes cultured per unit volume as well as the

amount of cumulus cover per oocyte. Olson *et al* (1991) found that while supplementation of the IVM medium with FSH (50 ng) and/or LH (1 μg) markedly enhanced cumulus expansion, it did not affect cleavage rate or early development. This is in agreement with the results of the present study where expansion was observed after inclusion of pFSH at any concentration studied and at pLH concentrations above 50 ng/ml.

There are conflicting reports in the literature regarding the necessity for and optimal concentrations of hormones and serum. The addition of gonadotropins, LH and/or FSH during IVM has been shown to improve the developmental potential for oocytes of the mouse (Jinno *et al*, 1989; Downs *et al*, 1991), rat (Shalgi *et al*, 1979; Vanderhyden and Armstrong, 1990), rabbit (Yoshimura *et al*, 1989), goat (Younis *et al*, 1991), sheep (Moor and Trounson, 1977; Staigmiller and Moor, 1984), and cow (Fukushima and Fukui, 1985; Brackett *et al*, 1989; Younis *et al*, 1989; Zuelke and Brackett, 1990). However, other authors have reported no effect of exogenous hormones (Fukui and Ono, 1989; Keefer *et al*, 1993). While the reasons for such discrepancies are not always clear they are probably partly related to differences in the presence/absence of serum and also due to differences in post-fertilization culture systems. The ability of protein-free defined culture medium to support hormonally enhanced bovine IVM has been demonstrated (Larson and Parks, 1990; Zuelke and Brackett, 1990; Coskun *et al*, 1991; Saeki *et al*, 1991; Ectors *et al*, 1992).

In the present study, the addition of both FSH and LH together to M199 or SOF depressed the blastocyst yield and hatching rate, following IVF. This effect was removed in the presence of serum. This is consistent with the known protective effect of serum and underlines the need to use a serum-free medium when testing the effects of exogenous substances. As pointed out by Downs *et al* (1991), the choice of macromolecular supplement is critical when testing the hormone responsiveness of isolated cumulus enclosed oocytes in culture. Most serum contains factors that suppress the hormone-induced response, emphasizing the importance of choosing medium supplements carefully.

When used individually, neither LH nor FSH, at any of the concentrations used, improved development over that of M199 alone. This suggests that in cases where the control was superior to M199 the beneficial effect was fully attributable to the presence of serum. It should be noted that in some experiments (see tables II and III) no beneficial effect of serum was observed. The reasons for the discrepancy are unclear but may be due to differences in responsiveness among oocyte batches. The reason for the failure to show a positive effect of hormones in the present study compared to other reports lies in part in the fact that in our system the use of unsupplemented M199 results in higher yields of embryos following IVF than reported by most authors, rather than a reduced effect of the added hormones. The reasons for this may include differences in media use (we have observed marked differences in batches of M199), the quality of the water used in media preparation, differences among batches of oocytes (perhaps relating to variations in time and conditions from ovary collection to oocyte submission to IVM) and conditions of post-fertilization culture.

It is perhaps worth pointing out that E_2 was not tested individually for 2 reasons. Firstly, due to its volatile nature it is likely that by the end of culture the amount of E_2 is negligible. Secondly, it has been shown (Bindal *et al*, 1988) that lipophilic impurities in commercial preparations of phenol red, a pH indicator dye commonly used in tissue culture media (*eg*, TCM 199) have weak estrogenic activity. As a result, the validity of assessing the effect of E_2 in such media is questionable.

It is interesting to note that rates of fertilization as assessed by cleavage after insemination were apparently unaffected by the presence of serum. This would suggest that zona hardening is not a problem in bovine oocytes as has been reported in mice (Downs *et al,* 1986) and rats (Vanderhyden *et al,* 1989; Zhang *et al,* 1991).

Despite a wide variety of protocols for the production of bovine embros, no one system has been proven to be consistently better. While a certain proportion of early developmental failure may be attributable to suboptimal culture conditions, it is clear from the work of many authors that germinal vesicle stage oocytes may not be developmentally equivalent. Indeed, one could question whether blastocyst yields in excess of 50% are realistically consistently achievable in practice given the morphological/qualitative heterogeneity of the raw material we began with (*ie* oocytes from slaughterhouse ovaries). As noted by Kruip *et al* (1979) no single culture system is likely to provide ideal conditions for oocytes obtained from every class of follicle. While oocytes for IVM are usually obtained from follicles 2–6 mm in size and at least 4–10 days away from any possible ovulation (Sirard *et al,* 1992), oocytes resuming meiosis *in vivo* originate from dominant follicles of about 15 mm in size (Pavlok *et al,* 1992). In addition, the IVM period lasts only 24 h, while the dominant follicle grows from 4 to 15 mm for approximately 5 d. It is likely therefore that developmental heterogeneity of chromosomally mature oocytes reflects intrinsic influences on the oocyte that occur differentially among follicles. Despite endless modifications of the IVM medium therefore, there may be a biological limit on the blastocyst yields achievable. It may well be that a prematuration treatment is necessary in order to allow the oocytes from smaller follicles, *ie* with 'interrupted' of folliculogenesis, to 'catch up' with those from larger follicles or those matured totally *in vivo.*

In conclusion to the present study, the identification of M199 as a completely defined medium capable of supporting the IVM of bovine oocytes in the absence of serum is an important step forward in the development of tools for the study of the specific metabolic requirements of maturing bovine oocytes. Studies in our laboratory are continuing to evaluate which serum components are responsible for the improved developmental competence of oocytes following IVM.

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